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Regulation of MSV and WDV virion-sense promoters by WDV nonstructural proteins: a role for their retinoblastoma protein-binding motifs

Angeles Muñoz-Martín,[a,b,1] Sylvie Collin,[b,c,1,2] Esther Herreros,[b,d] Philip M. Mullineaux,[c] María Fernández-Lobato,[d] and Carmen Fenoll[a,b,*]

[a] *Facultad de Ciencias del Medio Ambiente, Universidad de Castilla-La Mancha, E-45071 Toledo, Spain*
[b] *Departamento de Biología, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain*
[c] *John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK*
[d] *Centro de Biología Molecular "Severo Ochoa," Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain*

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Abstract

In this work we demonstrate that wheat dwarf virus (WDV) RepA can activate WDV and maize streak virus (MSV) virion (V)-sense expression in plant tissues. Rep alone does not have any effect on the silent WDV promoter and it represses the basal MSV promoter activity. MSV promoter activation by RepA depends on an intact RepA retinoblastoma protein (RB)-binding domain. Promoter repression by Rep also depends on this domain to some extent. Mutation of the RepA RB-binding domain has no effect on WDV promoter activation. The WDV promoter contains two sites that fit the consensus E2F-binding site. One, WDV1, binds human E2F-1 in one-hybrid assays in yeast. It also binds specifically to maize and wheat proteins in vitro and, when fused to a minimal 35S promoter, it confers responsiveness to RepA only when the RepA RB-binding domain and the WDV1 site are intact. In the whole WDV V-sense promoter context, mutations of this sequence have no effect, suggesting that additional sequences are important for RepA-mediated promoter activation.

Keywords: Mastrevirus; Transcriptional activation; V-sense promoters; geminiviruses; retinoblastoma; E2F; RepA; Rep

Introduction

Geminiviruses are plant DNA viruses whose very small genomes code for only a few proteins, none of them a DNA or an RNA polymerase (reviewed by Hanley-Bowdoin et al., 2000; Timmermans et al., 1994). Therefore, they must rely heavily on the host cell machinery for DNA replication and transcription to propagate themselves. Although geminiviral replication requires cellular factors for DNA synthesis, it takes place in nondividing cells (Lucy et al., 1996). For the begomovirus tomato golden mosaic virus (TGMV), replication in mesophyll cells correlates with Rep ability to efficiently bind the cellular retinoblastoma protein (RB) (Kong et al., 2000). It is not yet known how mastreviruses achieve replication and expression of their genomes in differentiated cells, but some clues can be inferred from the proteins they encode.

Geminiviruses from the genus *Mastrevirus*, such as wheat dwarf virus (WDV) and maize streak virus (MSV), have a genome composed of a single-stranded circular DNA molecule of ca. 2.6 kb which codes for four potential products. The virion strand (V-sense) codes for the coat and the movement proteins, and the complementary strand (C-sense) can produce the two nonstructural proteins RepA and Rep (reviewed by Hanley-Bowdoin et al., 2000; Palmer and Rybicki, 1998; Timmermans et al., 1994). For WDV, Rep is

* Corresponding author. Fax: +34-925-268840.
E-mail address: Carmen.Fenoll@uclm.es (C. Fenoll).
[1] A.M.-M. and S.C. have contributed equally to this work.
[2] Present address: Université Pierre et Marie Curie, UMR 7632 CNRS-Paris 6, PCMP, T53-E4, CC154, 4 Place Jussieu, 75252 Paris Cedex 05, France.

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essential for viral replication, while RepA is dispensable for this process (Collin et al., 1996; Schalk et al., 1989). RepA is necessary for WDV V-sense gene expression in wheat cell suspensions, Rep being required to achieve maximum expression (Collin et al., 1996).

The mechanisms by which Rep and RepA regulate V-sense gene expression are not known. RepA could have a direct role in transcriptional activation, since a C-terminal fragment of the MSV RepA can transactivate a GAL4-responsive promoter in yeast when fused to the GAL4 DNA-binding domain (Horvath et al., 1998). RepA could also exert its function through mechanisms involving interactions with cellular proteins. WDV and MSV Rep and RepA proteins contain a five-amino-acid motif (LXCXE) that allows their interaction with the cellular RB (Collin et al., 1996; Horvath et al., 1998; Xie et al., 1995). In mammals, RB binds transcription factors, such as members of the E2F family, that regulate the expression of genes necessary for G1 to S transition of the cell cycle (La Thangue, 1994). When RB is active, it disables E2F for transcriptional activation. RB inactivation by cyclin D-mediated phosphorylation or by oncoproteins releases E2F from its repressed state, thus allowing activation of E2F-responsive genes and cell proliferation (La Thangue, 1994; Slansky and Farnham, 1996). Animal tumor-inducing viruses code for oncoproteins that bind the active form of RB, often through an LXCXE motif, releasing E2F and other transcription factors from their repression. This allows DNA synthesis and cell-cycle progression, thus creating an adequate environment for viral replication. In addition, adenovirus promoters contain E2F-binding sites, making viral transcription E2F-dependent (Moran, 1993; Nevins, 1992).

Although WDV and MSV cannot be considered plant oncoviruses, they share some features with the animal tumor-inducing viruses, such as infecting differentiated cells and coding for RB-binding proteins. In nondividing cells, it could be hypothesized that Rep and/or RepA allow these geminiviruses to interfere with a cellular RB/E2F pathway, leading to transactivation of both viral promoters and plant genes required for DNA synthesis, in ways similar to those demonstrated for oncoviruses.

There is evidence that cell-cycle G1/S transition is conserved between animals and plants (reviewed by Gutiérrez, 1998; Inzé et al., 1999; Mironov et al., 1999; Potuschack and Doerner, 2001). Several plant homologs to CycD have been cloned (reviewed by Mironov et al., 1999). They are regulated by hormones, implicated in cell-cycle control (Cockcroft et al., 2000; Riou-Khamlichi et al., 1999), and interact with plant RB (Ach et al., 1997; Grafi et al., 1996; Nakagami et al., 1999; Xie et al., 1996; reviewed by de Jager and Murray, 1999; Durfee et al., 2000). Genes encoding plant E2F-like (Albani et al., 2000; de Jager et al., 2001; Kosugi and Ohashi, 2002; Mariconti et al., 2002; Ramírez-Parra et al., 1999; Sekine et al., 1999) and DP-like proteins (Magyar et al., 2000; Ramirez-Parra and Gutierrez, 2000) have also been cloned.

In this work, we explore the roles of WDV Rep and RepA and their RB-binding motifs in the regulation of both MSV and WDV V-sense gene expression.

Results

Activity of WDV and MSV V-sense promoters is modulated by the two WDV nonstructural proteins

Two different reporter plasmids (Fig. 1A) were tested by particle bombardment of maize leaves. The reporter constructs contained the MSV or WDV large intergenic region, plus flanking regions, fused to *GUS* in the V-sense.

The WDV promoter construct showed little or no *GUS* expression in this system, compared to the 35S–*GUS* construct, while the MSV promoter construct had a much higher activity (Fig 1B). This higher basal expression was not due to the additional viral sequences 5′ from the LIR present in the MSV promoter construct, since their deletion did not decrease promoter activity (A. Muñoz and C. Fenoll, unpublished results). To test the effect of WDV Rep and RepA on promoter activity, expression cassettes were made, consisting of Rep and RepA ORFs fused to a double 35S promoter (Fig. 1A), and they were cobombarded separately with the reporter plasmids. *GUS* expression from both reporters was enhanced by cobombardment with the RepA expression plasmid by a factor of about 2.3- and 40-fold for MSV and WDV, respectively. On the other hand, no activation was observed when the Rep-encoding plasmid was cobombarded with the WDV reporter construct (Fig. 1C). In the case of the MSV reporter, expression was abolished, indicating a repressor effect of WDV Rep.

To test whether these effects could be ascribed to an interaction of WDV Rep and RepA with RB, we made a mutation in the RB-binding motif of both proteins that changes the LXCXE motif to LXCXK and renders the proteins unable to interact with RB (Xie et al., 1995). The mutation abolished the RepA-dependent activation of the MSV V-sense promoter, but the mutated RepA was still able to activate the WDV V-sense promoter (Fig. 1B). This suggests that promoter activation by WDV RepA depends on the interaction of the protein with a cellular RB for the MSV promoter, but not for the WDV promoter. Although the slight decrease in the WDV V-sense promoter activity elicited by mRepA compared to RepA was not statistically significant, we cannot rule out some degree of participation of the RB pathway in the regulation of this promoter. The mutation also substantially affected MSV promoter repression by Rep (Fig. 1C).

Search for potential E2F-binding sites in WDV and MSV large intergenic regions

By analogy to adenoviruses, a role of the RB-binding activity of RepA may be to make E2F available for activa-

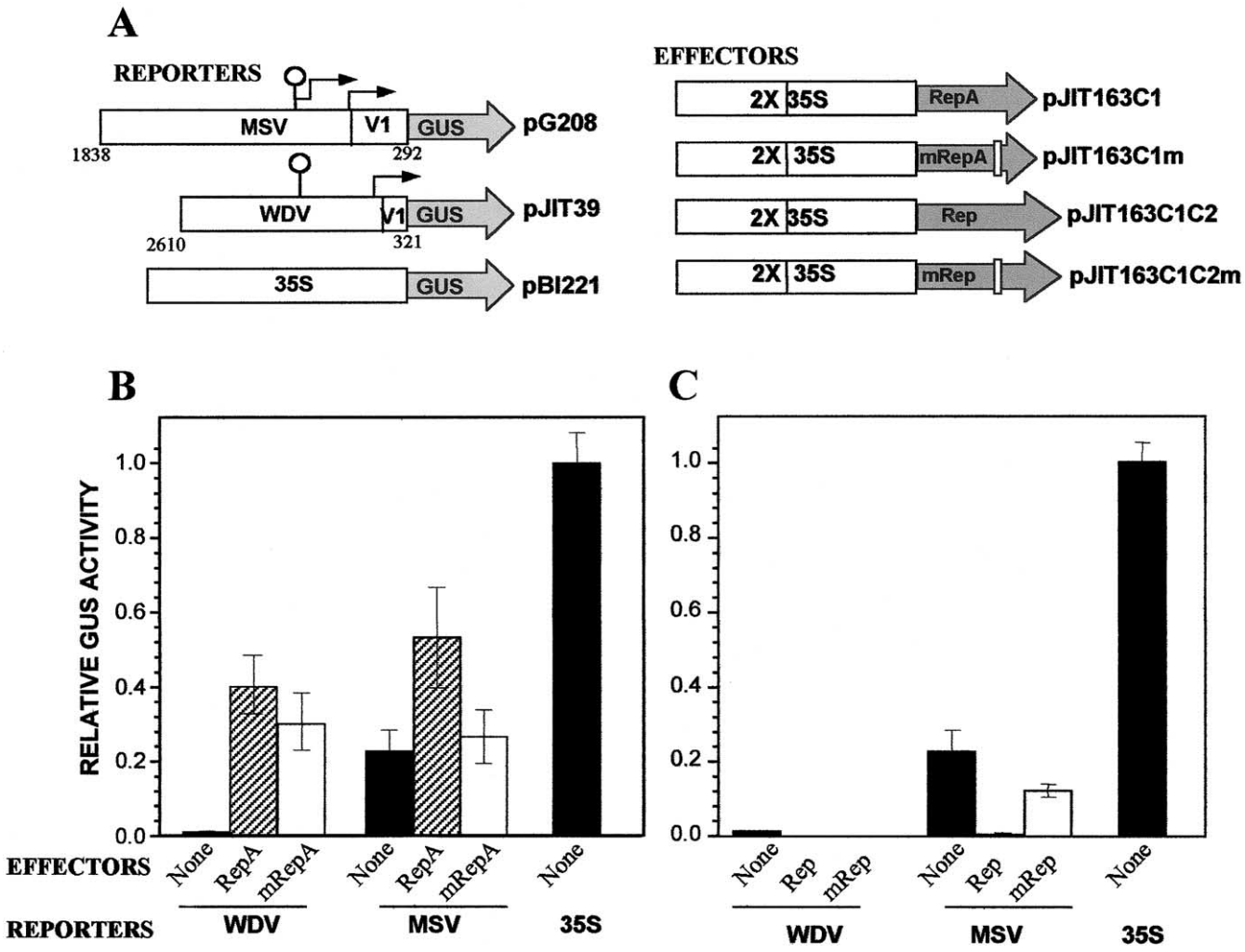

Fig. 1. Regulation of V-sense expression in WDV and MSV by RepA or Rep. (A) Diagram of the reporter and effector plasmids. The beginning of the transcripts and coordinates within the genome are indicated. Each reporter plasmid was bombarded into maize leaves, either alone or together with the effector constructs expressing RepA (B), Rep (C), or their mutated forms that do not bind RB. Each viral promoter activity was compared to that of the 35S promoter in pBI221. Bars indicate standard errors (SE). 35S promoter was not activated by RepA (not shown). The number of shots was 14 for the WDV promoter alone or with RepA and mRepA, 10 for the MSV promoter alone or with RepA and Rep, 5 for the MSV promoter alone or with mRepA and mRep, 5 for the WDV promoter with Rep and mRep, and 24 for the 35S promoter.

tion of both viral and S-phase-specific cellular promoters. We therefore looked for potential E2F-binding sites in the large intergenic regions of WDV and the related MSV, which contain the bidirectional promoters of the viruses. In MSV, no clear potential site was found. In WDV, two potential E2F-binding sites were found on the complementary strand between coordinates 2742–2729 (site WDV1) and 205–192 (site WDV2) (Fig. 2), both in a position consistent with a possible role in bidirectional transcription. Sequencing of several clones detected a one-base difference in the WDV2 sequence in comparison with the published WDV nucleotide sequence (MacDowell et al., 1986). The sequences showed 1-bp (WDV1) or 2-bp (WDV2) mismatches with respect to the consensus E2F-binding site (Black and Azizkhan, 1999; Slansky and Farnham, 1996). These changes did not correspond to mutations known to abolish the E2F binding (Hiebert et al., 1989; Ogris et al., 1993; Yee et al., 1987). WDV1 is identical to known functional mammalian binding sites found in the mouse b-*myb* and human thymidine kinase promoters (reviewed in Slansky and Farnham, 1996). Because WDV1 better matched a functional E2F-1-binding site, it was chosen for further study.

Interaction between WDV1 and human E2F-1

To assess the functionality of WDV1, its interaction with the human E2F-1 protein was tested using a one-hybrid system. A tandem trimer of the potential E2F-binding site WDV1 (Fig. 2) was placed in the promoter region of the yeast *HIS3* and *lacZ* genes, producing a reporter yeast strain (see Material and methods for details). This reporter yeast strain was transformed with a plasmid that coded for a fusion of human E2F-1 to the yeast GAL4 activation do-

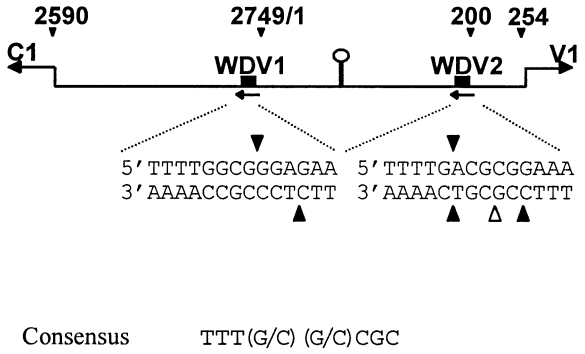

Fig. 2. Potential E2F-binding sites in the WDV large intergenic region (LIR). The diagram of the WDV LIR shows coordinates in the circular genome, indicated by vertical arrowheads. The hairpin loop conserved in all geminiviruses and involved in replication is marked, as is the beginning of ORFs V1 and C1. The two E2F-like-binding sites, WDV1 and WDV2, are indicated as black boxes. Their orientation is shown by arrows, and their nucleotide sequence is below. The one-base change in nucleotide sequence with respect to the previously published sequence is marked by an open arrowhead. The nucleotide changes with respect to the E2F consensus are indicated by black arrowheads. The E2F-binding site consensus sequence is also indicated.

main. Histidine auxotrophic colonies (Fig. 3A) were obtained, which indicates a positive interaction between the E2F-1 DNA-binding domain and the WDV1 trimer sequence. The activity of the second reporter gene, *lacZ*, was also induced, as demonstrated by the blue color of the colonies in a β-galactosidase filter assay (not shown). The reporter strain was transformed with control plasmids including the GAL4 activation domain either alone (pGAD) or fused to the DNA-binding domain of the tumor suppressor protein p53 (Fig. 3A). No histidine auxotrophs were recovered in either case, indicating that neither fusion protein could bind the WDV1 sequence, thus supporting the contention that the positive interaction between WDV1 and human E2F-1 is specific for the E2F-1 moiety. In addition, the E2F-1/GAL4 hybrid protein tested positive in a two-hybrid system for interaction with human and maize RB proteins (Fig. 3B).

Interaction of WDV1 with maize and wheat nuclear proteins

Bearing in mind that WDV1 strongly resembles an E2F-1-binding site, and the fact that human E2F-1 binds to WDV1 in yeast, we tested if wheat, the natural host for WDV, contains nuclear proteins that can bind WDV1. Nuclear extracts were made from actively growing wheat cell suspensions and tested in electrophoretic mobility shift assays using as a probe the radiolabeled WDV1 trimer (Fig. 4A). The WDV1 trimer was also challenged with nuclear extracts from maize, in which WDV can replicate and transcribe its genome (Timmermans et al., 1992) and the V-sense WDV promoter is responsive to RepA (this work). The specificity of the proteins for WDV1 was tested by competition experiments using a large molar excess of unlabeled oligonucleotide in the binding mixture (Fig. 4A). Unlabeled WDV1 displaced the labeled WDV1 probe very efficiently from complexes made with both wheat and maize nuclear proteins (Fig. 4A; lanes 2 and 6–8), an indication that these proteins have a high affinity for the WDV1 sequence. However, equivalent amounts of an oligonucleotide with unrelated sequence (lanes 3–5) had little competitor effect.

Because the WDV1 trimeric oligonucleotide contained sequences other than the core E2F recognition site, introduced by concatemerization, a shorter oligonucleotide representing just one such site (wtWDV1) was designed to perform similar assays. When used as a probe, wtWDV1 was able to bind nuclear proteins from wheat (Fig. 4B). A 100-fold excess of the unlabeled oligonucleotide displaced the probe from the complexes (Fig. 4B, lane 3). Wild-type WDV1 also formed complexes with maize proteins, which disappeared with a 500-fold excess of unlabeled oligonucleotide (Fig. 4B, lane 3). In both cases, 3 μg of poly(dA-dT), a nonspecific double-stranded competitor, did not displace the probe (lane 6).

To assess the specificity of this DNA–protein interaction, a mutant version of the oligonucleotide (mWDV1) was made. It harbored three-point mutations in the central GC core of the sequence, previously shown to abolish E2F binding in animal systems (Hiebert et al., 1989; Ogris et al., 1993; Yee et al., 1997). mWDV failed to compete efficiently for protein binding with the wild-type sequence even at a 500-fold molar excess (Fig. 4B, lane 5 in the maize panel). These results indicate that wheat and maize cell nuclear extracts contain proteins that recognize the E2F-like binding sequence in WDV1 in a very specific manner.

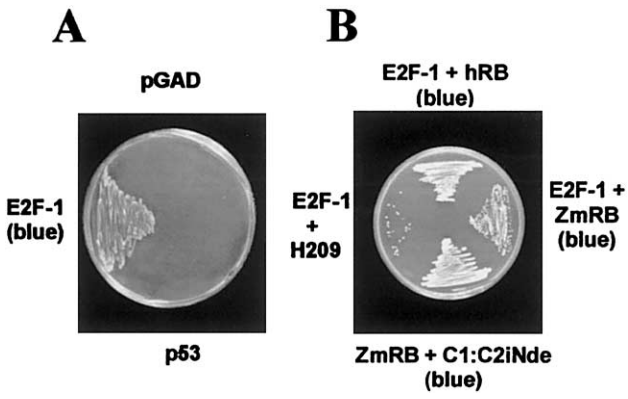

Fig. 3. Functional assay for the interaction of human E2F-1 with the WDV1 sequence and maize RB in yeast. (A) Interaction of E2F-1 with the WDV sequence. YSCi10 yeast strain, containing a WDV1 trimer fused to *HIS3* and *LacZ* coding sequences, was transformed with plasmids expressing human E2F-1, p53, or neither (pGAD) fused to the GAL4 activation domain and checked for growth on selective medium and β-galactosidase activity. (B) Two-hybrid assay using the Y153 yeast reporter strain. The plasmids expressed fusions of the GAL4 DNA-binding domain to either maize RB (ZmRB) or human RB (hRB) or an inactive form of human RB (H209) and fusions of the GAL4 activation domain to either human E2F-1 (E2F-1) or WDV RepA (C1:C2iNde).

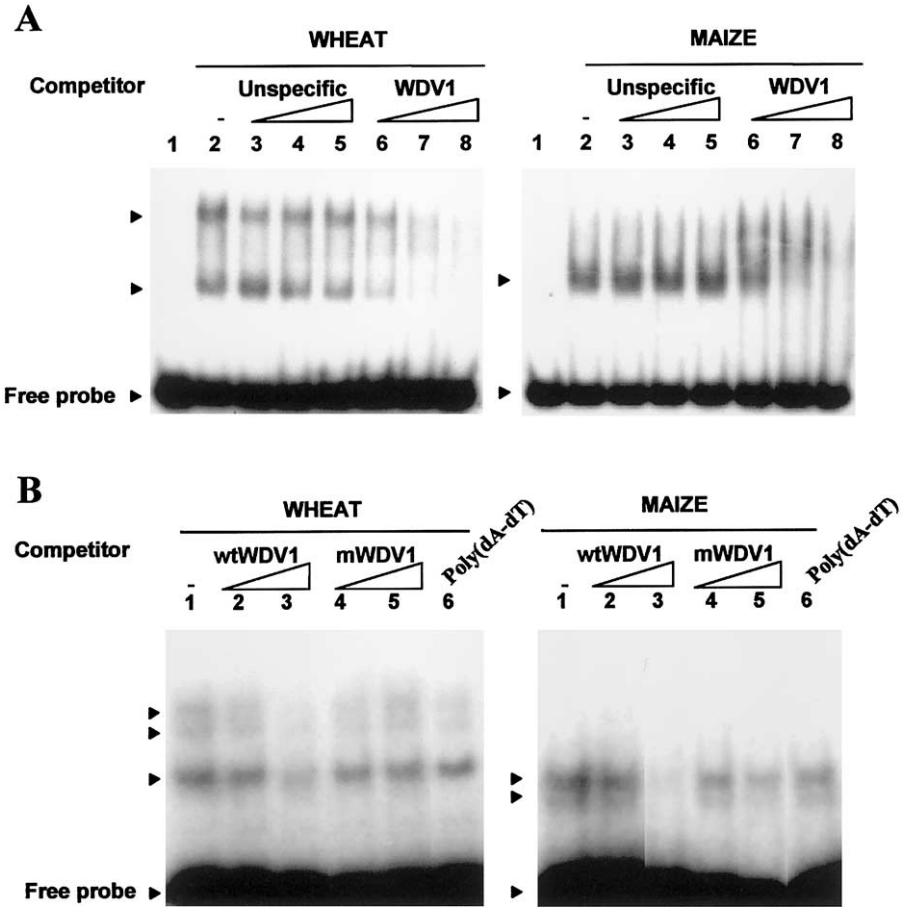

Fig. 4. Binding of plant nuclear proteins to WDV promoter-derived sequences. Electrophoretic mobility gel shift assays were performed with wheat and maize nuclear protein extracts (18 μg), using the end-labeled double-stranded oligonucleotide representing the trimer of WDV1 as a probe and 1 μg of poly(dI-dC) as nonspecific DNA competitor. (A) Binding reactions were performed in the presence of a 10-, 100-, or 500-fold molar excess of either an unlabeled unrelated oligonucleotide (lanes 3–5) or unlabeled WDV1 trimer oligonucleotide (lanes 6–8). Lane 1, no protein extract; lane 2, no competitor DNA. (B) Competition assays using synthetic oligonucleotides representing a single wtWDV1 sequence as a probe. Competitor DNA was none (lane 1), wtWDV1 (lanes 2 and 3), mWDV1 (lanes 4 and 5), or 3 μg poly(dA-dT) (lane 6). Amounts of competitor relative to the probe were 10-fold (lanes 2 and 4) and 100-fold (lanes 3 and 5) for the wheat extracts and 10-fold (lanes 2 and 4) and 500-fold (lanes 3 and 5) for the maize extracts. Arrowheads indicate the free probe and the retarded DNA–protein complexes.

WDV1 confers RB-dependent RepA-responsiveness to a minimal promoter in maize

To test whether the WDV1 ability to bind human E2F-1 and plant nuclear proteins had a functional significance, we fused the WDV1 trimer (Fig. 2A) to a minimal 35S promoter (deleted up to position −46) controlling *GUS* (Fig. 5A) and bombarded the chimeric construct into maize leaves. The plasmid produced a barely detectable GUS activity (Fig. 5B).

When the reporter plasmid was cobombarded with a construct expressing the WDV RepA protein under the control of the double 35S promoter (Fig. 1A), we detected an 18-fold enhancement in GUS activity (Fig. 5B). Cobombardment with a plasmid expressing the mutant RepA protein under the control of the double 35S promoter resulted in only a 2-fold enhancement of *GUS* expression from the reporter plasmid (Fig. 5B). A new reporter plasmid was made replacing the WDV1 trimer with another trimer harboring the same mutations that abolished plant protein binding in mWDV1 (see previous section). When this mutated construct (Fig. 5A) was bombarded into maize leaves, it produced a very low background activity that was not enhanced by cobombardment with the plasmids expressing either the wild-type or the mutated RepA (Fig. 5B). Therefore, the same viral sequence that binds human E2F-1 and plant nuclear proteins can be activated by RepA in an RB interaction motif-dependent manner.

WDV1 mediates E2F-1, but not RepA-dependent, activation of the WDV V-sense promoter

To assess the role played by WDV1 in the context of the complete WDV promoter, we replaced the native WDV1 sequence by the mWDV1 sequence in pJIT39, to make pJIT39m1, and performed transient expression assays by particle bombardment in developing maize leaves. The

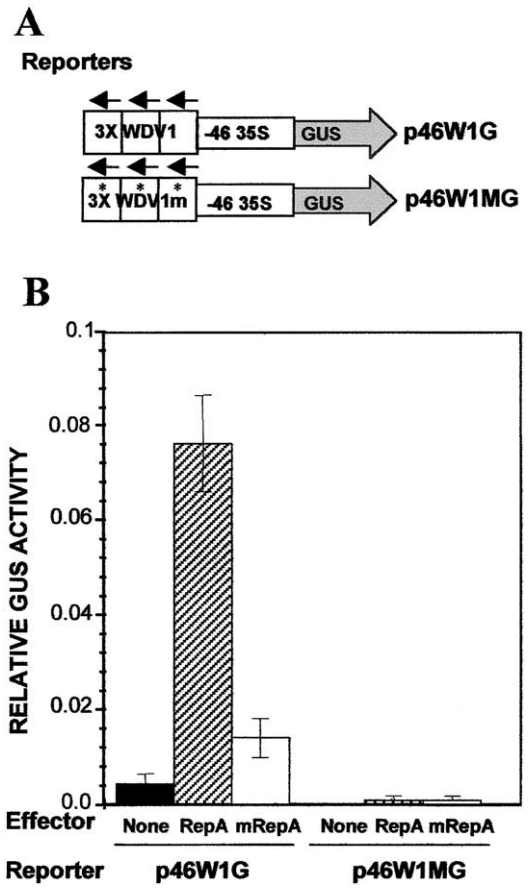

Fig. 5. Regulation of WDV1-dependent expression by RepA in maize leaves. (A) Diagram of the reporter plasmids used for the bombardments. The *GUS*-based reporter plasmid (p46W1G) includes the trimeric WDV1 sequence fused to the 5′ end of the minimal (−46/+1) 35S promoter. p46W1MG contains three point mutations in the GC central core of the WDV1 sequence. The effector plasmids carrying a double 35S promoter driving the expression of RepA or a mutant RepA that does not bind RB were described in the legend to Fig. 1A. (B) The reporter plasmids were bombarded into maize leaves either alone or together with each of the effector plasmids. All values are relative to the GUS activity obtained with the 35S promoter (pBI221) in each independent experiment as described in Material and methods. Bars, SE. The number of shots was 17 for p46W1G alone or with RepA, 9 for p46W1G with mRepA, 5 for p46W1MG alone or with RepA or mRepA, and 21 for the 35S promoter.

WDV promoter mutated in the WDV1 sequence fully retains RepA inducibility (Table 1), but no activation was found using human E2F-1 as an effector. The native WDV promoter was able to respond to human E2F-1 with a limited, but statistically significant, activation (Table 1). Therefore, while the WDV1 sequence is required for E2F-1 activation, it seems not to be required for the RepA-mediated activation of the complete WDV promoter.

Discussion

This work was motivated by the hypothesis that the geminivirus RepA protein might activate viral V-sense expression by interfering with a cellular pathway for the regulation of gene expression involving the plant equivalents of RB and/or E2F.

In the first series of experiments using maize, we have shown that WDV RepA activates V-sense gene expression in WDV, but apparently in an RB-independent manner. The mutation we introduced in RepA has previously been demonstrated to impair RB binding in a two-hybrid assay (Xie et al., 1995). In maize, this mutation has no statistically significant effect on the ability of the protein to activate V-sense expression in WDV, albeit it elicited a slightly lower promoter activity than the unmutated RepA. In parallel, we have identified two putative E2F-1-binding sites in the WDV promoter. The one chosen for study, because of its close resemblance to known E2F-binding sites, is functional in maize, since it confers on a minimal promoter human E2F-1- and RB-binding motif dependency for expression. However, the site is not necessary for RepA activation of the V-sense promoter, although RepA-elicited activation was slightly lower in mWDV than in the wild-type promoter. RepA-mediated WDV V-sense expression is, therefore, RB- and E2F-independent in our system, although it cannot be excluded that in other hosts and cell types, or at different developmental stages, it could be RB- and/or E2F-dependent.

The mutated RepA is still fully able to activate WDV V-sense expression, implying either that it is the direct activator of expression, thus functioning as a transcriptional factor, or that it acts indirectly, via unidentified factors. RepA is a modular protein with several putative functional domains, and it can bind sequences in the WDV large intergenic region between nucleotides −193 to −116 and +74 to +135, at least in vitro (Castellano et al., 1999). In our system, this binding might play a prevalent role in the

Table 1
Effects of RepA or human E2F1 on the activity of wild-type and mutated WDV V-sense promoters

Reporter	Effector	Relative Gus activity[a]	N[b]
pJIT39 (wtWDV1)	—	0.012 ± 0.003[1]	14
	E2F1	0.032 ± 0.009[2]	12
	RepA	0.400 ± 0.132[3]	14
pJIT39m1 (mWDV1)	—	0.009 ± 0.006[4]	4
	E2F1	0.004 ± 0.001[5]	4
	RepA	0.340 ± 0.077[6]	13

Note. Maize leaves were bombarded with reporter plasmids expressing GUS under the control of wild-type WDV V-sense promoter (pJIT391) or a version containing the same point mutations in the WDV1 sequence that abolish binding of nuclear proteins (pJIT39m1). The reporters were tested alone or cobombarded with expression cassettes for the human E2F1 or WDV RepA, under double 35S promoter control, as effector plasmids.

[a] Relative GUS activity is measured as the number of blue spots in each assay relative to the blue spots obtained with the 35S promoter ± SE.

[b] *N* is the number of shots per construct combination. Using the *t* test, the following values are significantly different ($P < 0.05$): means between 1 and 2 ($P = 0.037$), 1 and 3 ($P = 0.001$), 4 and 6 ($P = 0.001$); the differences between 4 and 5 ($P = 0.386$) and 3 and 6 ($P = 0.745$) are not significant.

WDV V-sense promoter, thus obscuring other possible mechanisms such as an RB motif-mediated activation, because WDV RepA should be better adapted for interactions with wheat than with maize RB proteins. The MSV RepA possesses a domain capable of activating transcription in yeast (Horvath et al., 1998), and sequence homology between the MSV and WDV RepA proteins suggests that WDV RepA could also be responsible for such a direct promoter activation.

Alternatively, RepA could act indirectly through interactions with other cellular proteins. In this respect, the activator proteins of begomoviruses (AC2, AL2, or TrAP) activate V-sense gene expression without binding any promoter sequence in a specific way, but seemingly interacting with cellular proteins by unknown mechanisms (Hartitz et al., 1999; Sunter and Bisaro, 1997). The cellular partners for RepA remain to be determined. The only cellular proteins, other than RB, known to interact with RepA are the GRAB proteins (Xie et al., 1999). They have a NAC domain similar to that present in proteins involved in plant meristem development and in senescence (Aida et al., 1997). Although GRAB1 and GRAB2 overexpression interferes with WDV DNA replication in wheat cell suspensions (Xie et al., 1999), no data link GRAB proteins with V-sense gene expression as yet.

Our data suggest that, for the expression of the chimeric WDV1/−46 35S promoter, the RB-binding motif-mediated pathway involves plant transcription factors whose target sequences are E2F-like binding sites, that is, putative plant E2F-like factors. This is not unexpected, since plant gene promoters regulated by E2F-like-binding sites have been reported (Chaboute et al., 2000, 2002; Egelkrout et al., 2001; Kosugi and Ohashi, 2002). One of them, the *Nicotiana benthamiana* PCNA promoter, is upregulated in mature leaves infected by the begomovirus TGMV, but only when the E2F-binding site is intact (Egelkrout et al., 2001). In two cases, these E2F-like-binding sites are targeted by plant E2F-like transcription factors (Chaboute et al., 2002; Kosugi and Ohashi, 2002). Using the *Arabidopsis* genome database, the presence of E2F-like-binding sequences has been reported in the 5′ upstream regions of predicted genes associated with DNA synthesis, replication, mismatch repair, and the cell cycle. The sequences are classified in two conserved classes: tTTCCCGC and TTTGGCG(G/C) (Kosugi and Ohashi, 2002). The WDV1 sequence found in the complementary strand is similar to the first class and, in the sense strand, it matches the second (see Fig. 2.)

Other possible candidate plant transcription factors that might activate the chimeric WDV1/−46 35S promoter are Sp1-like proteins. In mammals, some members of the Sp-1 family are regulated by RB (Chen et al., 1994; Kim et al., 1992), and they bind specifically to GC-rich sites whose sequence (CCCGCC) is identical to the central GC core present in the WDV1 sequence. This GC-rich sequence is disrupted in the WDV1-mutated version of the chimeric promoter that fails to respond to RepA. In animal systems, cellular and chimeric promoters containing E2F-binding sites can be regulated by a combination of Sp1 and E2F factors (Karlseder et al., 1996; Lin et al., 1996). Whatever the transcription factors involved in the activation of the WDV1/−46 35S promoter be, our results demonstrate that an RB-mediated pathway for the regulation of gene expression is operative in plants.

Contrary to what happens with WDV-based constructs, in maize leaf cells WDV RepA is able to activate expression from the MSV V-sense promoter in a strict RB-binding domain-dependent manner and in the absence of other viral proteins. This is the first evidence that a plant promoter can be regulated by RB, although the relevance of this activation in the infection process remains to be tested. In this case, we have no hints as to what plant proteins might play a role in activation, since no recognizable E2F-binding site is present in the MSV promoter. However, many putative transcription factor-binding sites can be found in the promoter using the TRANSFAC program (Wingender et al., 2000), among them AP1 (c-Fos, c-Jun heterodimer-binding site) and MyoD, although the corresponding plant homologs have not yet been described. It is not known whether the corresponding transcription factors are regulated via sequestering by RB, but such a system is not uncommon. In oncoviruses such as SV40, multiple mechanisms of transcriptional regulation mediated by the large T- and small T-antigens have been described to act through RB and its interactions with several transcription factors, namely Spl, c-Fos, c-Jun, MyoD, and TBP (Moens et al., 1997). The RB-mediated pathway disclosed in our experimental system may not be the only route for the regulation of MSV V-sense promoter expression during viral infection. For instance, the MSV RepA protein might be able to interact with the promoter, while the heterologous WDV RepA might not.

Surprisingly, overexpression of WDV Rep had a repressor effect on MSV V-sense promoter expression. This repression was lower when the interaction of Rep with RB was abolished by a specific mutation, but little can be drawn from this result. The question arises as to whether Rep could alter the expression of specific cellular genes or even if it could be a general transcriptional repressor. This would fit the fact that we have not been able to regenerate WDV Rep-overexpressing transgenic tobacco plants. Other groups have also reported difficulties in obtaining plants that expressed Rep from monopartite begomoviruses (Bendahmane and Gronenborn, 1997; Dry et al., 2000).

By studying the effects of two viral proteins, WDV RepA and Rep, on V-sense expression from two mastreviruses in maize cells, we have been able to show that distinct pathways for gene expression may exist. One pathway that operates in the MSV promoter strictly depends on an RB-dependent activation route, although the involvement of an E2F-like factor remains to be determined; the other, which operates in the WDV promoter, depends neither on RB nor on a functional E2F-like-binding site present in the bidirectional promoter sequence. The possibility that the RB-de-

pendent pathway operates in the WDV V-sense promoter when expressed in different plant species and cell types or during different developmental stages remains to be addressed. Conversely, MSV RepA might be able to activate the MSV V-sense promoter by an RB-independent route. Indeed, RepA proteins, in spite of their conserved sequences in related mastreviruses, seem to target different cellular pathways in different geminiviruses. For example, the same mutation in the RB interaction motif abolishes replication in WDV (Xie et al., 1995), but it has no detectable effect on bean yellow dwarf virus replication (Liu et al., 1999). In this context, the different contribution of the RB interaction domain of WDV RepA to expression from different promoters found by us is hardly surprising. Dynamic interactions between Rep and RepA themselves and with specific promoter regions, as well as different viral protein ratios, might also contribute to the spatial–temporal regulation of V-sense expression during the infection cycle. Future research on the mechanisms that underlie the interaction of geminiviral proteins with RB will be helpful in providing new insights not only into the comprehension of geminivirus infection but also into the mechanisms that control cell division and differentiation in plants.

Materials and methods

Constructs used in transient expression assays

Viral sequences are derived from the MSV Nigerian isolate (accession No. X0163) and WDV Czech Republic isolate (accession No. X02869).

To obtain pG208, the 1168-nt *Hin*dIII–*Xba*I fragment from pCF208 (Fenoll et al., 1988) was fused to the GUS coding sequence in pGUS1 (Plant Genetic Systems, Holland) digested with *Hin*dIII and *Sal*I. pJIT39 was constructed by ligating the 456-nt *Hpa*II fragment from pJIT34 (Woolston et al., 1988) to *Sma*I-linearized M13mp18 (Yanisch-Perron et al., 1985). The *Kpn*I–*Pst*I internal fragment was cloned into pJIT75 (Guerineau et al., 1992) digested with *Kpn*I and *Sma*I. pJIT39m1 was obtained by site-directed mutagenesis by conventional PCR techniques (Ausubel et al., 1997), using as primers carrying the mutations (shown in lower case) the following oligonucleotides: CCCTGTTCTCaaaCCAAAACCTGC, GCAGGTTTTGGtttGAGAACAGG, GGAATAAGGGCGACCGGA, and GATTTCACGGGTTGGGGTTT. The native sequence in pJIT39 was replaced by the PCR fragment harboring the mutations, digested with *Kpn*I and *Sal*I. Mutated clones were confirmed by sequencing.

To obtain pJIT163C1, the intermediate plasmid pSTAGEIIIA was constructed. pSTAGEIIIA is a pBlueScript derivative that contains a *Not*I–*Hin*dIII fragment from pWDV2 (Hofer et al., 1992) including the whole C1 and C2 ORF sequences. The 835-nt *Nco*I–*Nde*I fragment from pSTAGEIIIA, including the RepA coding sequence, was fused to pJIT163*Bgl*II (Creissen et al., 1995) previously linearized with *Nco*I and *Sma*I.

pJIT163C1m was obtained by site-directed mutagenesis performed by conventional PCR techniques (Ausubel et al., 1997) on the RepA sequence contained in pSTAGEIIIA, which produced an E-197 to K substitution. The oligonucleotides used as specific primers carrying the relevant mutations (indicated in lower case) are OM1, CGTTGACGTCATATGTTGTGAAATCAACTA, which is homologous to nts 819–849 in the RepA coding sequence; OM2, CACTCATTTGCCATaAGACCATTGAAAGC-; OM3, GCTTTCAATGGTCTtATGGCAAATGAGTG, both homologous to nts 578–606 in the RepA coding sequence; and OM4, CGTGCAGAACAAGCTTCGTGCTTCCATCAC, homologous to nts 190–219 in the RepA coding sequence. The 636-nt *Hin*dIII–*Nde*I mutant fragment obtained was used to replace the native one in pSTAGEIIIA. The mutation was verified by DNA sequencing. The final construct (pJIT163C1m) includes the *Nco*I–*Nde*I fragment in the RepA mutant protein, which was fused to pJIT163 (Guerineau et al., 1992) previously digested with *Hin*dIII–*Sma*I.

pJIT163C1C2 was constructed by cloning the 1216-nt intronless Rep sequence from pSTAGEIIIAi-, digested with *Nco*I and *Ssp*I, into pJIT163 digested with *Nco*I and *Sma*I. pSTAGEIIIAi- is identical to pSTAGEIIIA except that the wild-type sequence coding for C1 and C2 has been replaced by the intronless C1:C2 cDNA (Collin et al., 1996; Dekker et al., 1991).

pJIT163C1C2m was obtained by PCR similarly to pJIT163C1m, using the same oligonucleotides on pSTAGEIIIAi-. The mutated Rep coding sequence was obtained by digesting the PCR product with *Nco*I and *Ssp*I and introduced in pJIT163 digested with *Nco*I and *Sma*I.

pJIT163E2F was obtained by ligating the 1405-nt *Eco*RI–*Hin*dIII fragment from pGAD424XhoE2F-1 (described below) into *Eco*RI-linearized pJIT163.

pWDV1GUS is a pGUS1 derivative including the *Hin*dIII–*Pst*I trimeric WDV1 sequence from pSC1H10 (described below). The (−46/+1) 35S core promoter was obtained by PCR using pBI221 (Jefferson, 1987) as a template and AACAACCCTGCAGCAAGACCCTTCCT and GGTACCCATGGACATAAGGGACTGAC as primers. The PCR product was digested with *Pst*I and *Nco*I and introduced into pWDV1GUS to produce p46W1G. p46W1MG was obtained by replacing the *Hin*dIII–*Pst*I trimeric WDV1 sequence in p46W1G with the double-stranded oligonucleotide ACCTTGATATCGAATTC-$(TTTTGGTTTGAGAA)_3$GAATTCCTGCA, corresponding to a mutated tandem trimer of WDV1.

Transient expression assays

Maize seeds (kindly provided by DK Seeds, Spain) were germinated for 24 h in the dark on wet filter paper and under sterile conditions. Seedlings were subsequently grown for 7–10 days under a 16-h photoperiod, at 24°C. Leaves were collected and laid in a petri dish with nutrient medium (MS, 3% (w/v) sucrose, 0.6% (w/v) agar, and 0.4 M mannitol) for

bombardment. Plasmid DNA was coprecipitated with 1-μm-diameter gold particles. Bombardments were made in a PDS1000/He (Bio-Rad) apparatus, at 1100 psi as described (Klein et al., 1988), with some modifications (M. Menossi and J.A. Martínez-Izquierdo, unpublished) for maize tissue bombardment. Bombarded leaves were kept under the same growth conditions for 24 h and stained for histochemical GUS detection as described (Jefferson, 1987). Blue spots were quantified from at least five independent shots each comprising three different leaves that always covered the same area (a 3-cm-diameter circle) in the plate, from several independent experiments. All data were normalized in relation to the number of spots obtained with pBI221 in each independent experiment.

Yeast one-hybrid and two-hybrid assays

The Matchmaker one-hybrid system (Clontech, Palo Alto, CA) was used for testing the binding properties of the putative E2F-binding site WDV1. The yeast reporter strain was constructed as follows. The double-stranded oligonucleotide AATTC(TTTTGGCGGGAGAA)$_3$G, corresponding to a tandem trimer of WDV1, was first cloned into *Eco*RI-digested pBluescript SK+ to make pSC1H10. The insert was sequenced to determine the orientation of the polylinker and excised either with *Xho*I and *Sma*I for insertion in the promoter region of the *lacZ* reporter gene in pLacZi or with *Eco*RV and *Sac*I for insertion in the promoter region of *HIS3* in pHISi. The resulting plasmids were linearized and used for transformation of the yeast acceptor strain by integration into the genome. Screening of the transformants was carried out according to the manufacturer's instructions for selection of the clones with the lowest *HIS3* and *lacZ* background activity. The selected strain, pYSCi10, showed undetectable lacZ activity, and growth without histidine was residual in 25 mM 3-aminotriazole. This concentration was therefore chosen for further selection of the positive clones during the binding experiments.

Construction of pGAD424Xho (pGAD) plasmid was carried out by inserting a *Xho*I linker (5′-AATTCCGCTCGAGCG-3′) between the *Eco*RI and *Bam*HI sites in the polylinker region of pGAD424. Human *E2F-1* cDNA was excised from pCMVE2F-1 (Kaelin et al., 1992), kindly provided by D.M. Livingston, using *Eco*RI and *Bam*HI, and ligated into pGAD restricted with *Sal*I and *Bam*HI to produce pGADE2F1 (abbreviated as E2F1). Transformation of the reporter yeast and screening of the yeast transformants was carried out as described in the manufacturer's instructions.

The two-hybrid assays were performed as previously described (Collin et al., 1996). Yeast cells were transformed with plasmids containing fusions of the GAL4 activation domain to WDV RepA (pACTC1:C2iNde, abbreviated C1C2iNde; Collin et al., 1996) or human E2F-1 protein (E2F-1), together with plasmids containing fusions of the GAL4 DNA-binding domain to either a wild-type (pASRb2) or a mutant (pASH209, abbreviated H209) human RB (Durfee et al., 1993) or to a partial cDNA from a maize RB homolog that includes both A and B pocket domains of the protein (pAS/zRb(RV-C, abbreviated ZmRB; Ach et al., 1997), kindly provided by T. Durfee (UC Berkeley). The yeast strain Y153 and the transformation procedure were as described by Durfee et al. (1993). Screening of the yeast transformants was carried out as described by Collin et al. (1996).

Gel retardation assays

Growth of *Zea mays* BMS (maize) and *Triticum monococcum* (wheat) cell suspension lines was as described previously (Fenoll et al., 1988; Hofer et al., 1992). Crude nuclear protein extracts were prepared from freshly collected cell suspensions 3 to 4 days postsubculture according to previously described methods (Fenoll et al., 1988, 1990).

The DNA fragment containing the putative E2F-binding site WDV1 used in gel retardation assays was recovered from pSC1H10, digested with *Eco*RI, and end-labeled by filling-in with [α^{32}P]dATP and ϕ29 DNA polymerase 3′–5′ Exo$^-$ (Bernad et al., 1989), kindly provided by M. Salas (CBMSO, Madrid, Spain). The labeling reaction was conducted as described by Esteban et al. (1992).

Synthetic oligonucleotides representing the wild-type WDV1 sequence (wtWDV1) in the WDV promoter (AATTCCCTGTTCTCCCGCCAAAACCTGC and AATTCGCAGGTTTTGGCGGGAGAACAGGG) were annealed and end-labeled as described. The mutated version (mWDV1) used as unlabeled competitor was obtained by annealing oligonucleotides AATTCCCTGTTCTCAAACCAAAACCTGCG and AATTCGCAGGTTTTGGTTTGAGAACAGGG.

Gel retardation assays were performed as previously described (Fenoll et al., 1988), except for the addition of a proteinase inhibitor mixture (leupeptin, pepstatin A, antipain, and chemystatin, 0.1 mg/mL each). Nonspecific competitor was a synthetic double-stranded oligonucleotide representing concatemers of the ACCGGGCCGG box, which binds specific proteins from maize extracts (Fenoll et al., 1990). They were added to the binding mix prior to the nuclear protein extract.

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